

QIAGEN® PCR Cloning Handbook

For

QIAGEN PCR Cloning Kit

QIAGEN PCR Cloning^{plus} Kit

April 2001



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Kit Contents

QIAGEN PCR Cloning Kit

QIAGEN PCR Cloning Kit	(10)	(40)
Catalog No.	231122	231124
Ligation Master Mix, 2x	50 µl	200 µl
pDrive Cloning Vector	0.5 µg	2.0 µg
Distilled water	1.7 ml	1.7 ml

QIAGEN PCR Cloning^{plus} Kit

QIAGEN PCR Cloning ^{plus} Kit	(10)	(40)
Catalog No.	231222	231224
BOX I		
Ligation Master Mix, 2x	50 µl	200 µl
pDrive Cloning Vector	0.5 µg	2.0 µg
Distilled water	1.7 ml	1.7 ml
BOX II		
QIAGEN EZ Competent Cells	10 tubes, 50 µl each	40 tubes, 50 µl each
SOC medium	2 x 1.9 ml	6 x 1.9 ml

Shipping Conditions

QIAGEN® PCR Cloning Kits are shipped on dry ice. Please verify upon shipment arrival that dry ice is still present in the shipping container. The QIAGEN PCR Cloning^{plus} Kit is packaged in two boxes: Box I contains reagents for ligation of PCR products into the pDrive Cloning Vector, and Box II contains QIAGEN EZ Competent Cells and SOC medium.

Storage and Stability

The QIAGEN PCR Cloning Kit and Box I of the QIAGEN PCR Cloning^{plus} Kit (containing Ligation Master Mix and pDrive Cloning Vector) should be stored at –20°C in a constant-temperature freezer or at –70°C **immediately** upon receipt. Box II of the QIAGEN PCR Cloning^{plus} Kit (containing QIAGEN EZ Competent Cells) should be stored at –70°C or below **immediately** upon receipt. Care should be taken to avoid thawing of competent cells. When stored under these conditions and handled correctly, the kit components can be kept at least until the expiration date (see inside of kit lid) without showing any reduction in performance.

Product Use Limitations

QIAGEN PCR Cloning Kits are developed, designed, and sold for research purposes only. They are not to be used for human diagnostic or drug purposes or to be administered to humans unless expressly cleared for that purpose by the Food and Drug Administration in the USA or the appropriate regulatory authorities in the country of use. All due care and attention should be exercised in the handling of the materials described in this text.

Product Warranty and Satisfaction Guarantee

QIAGEN guarantees the performance of all products in the manner described in our product literature. The purchaser must determine the suitability of the product for its particular use. Should any product fail to perform satisfactorily due to any reason other than misuse, QIAGEN will replace it free of charge or refund the purchase price. We reserve the right to change, alter, or modify any product to enhance its performance and design. If a QIAGEN product does not meet your expectations, simply call your local Technical Service Department or distributor. We will credit your account or exchange the product — as you wish.

A copy of the QIAGEN terms and conditions can be obtained on request, and is also provided on the back of our invoices. If you have questions about product specifications or performance, please call one of the QIAGEN Technical Service Departments or your local distributor (see inside front cover for contact information).

Technical Assistance

At QIAGEN we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in molecular biology and the use of QIAGEN products. If you have any questions or experience any difficulties regarding QIAGEN PCR Cloning Kits, or QIAGEN products in general, please do not hesitate to contact us.

QIAGEN customers are a major source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at QIAGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

For technical assistance and more information, please call one of the QIAGEN Technical Service Departments or your local distributor (see inside front cover for contact information).

Safety Information

Material safety data sheets (MSDSs) are available online in convenient and compact PDF format at www.qiagen.com/ts/msds.asp. You can find, view, and print the MSDS for each QIAGEN kit and kit component.

Introduction

QIAGEN PCR Cloning Kits combine the latest ligation technology with a unique combination of time-saving features for fast, easy, and highly efficient cloning of PCR products generated using *Taq* and other non-proofreading DNA polymerases. The pDrive Cloning Vector provides superior performance through UA-based ligation and allows easy analysis of cloned PCR products. The Ligation Master Mix contains all other reagents and cofactors required for optimal ligation in a convenient, pre-mixed format. QIAGEN EZ Competent Cells, provided in ready-to-use aliquots in the QIAGEN PCR Cloning^{plus} Kit, are capable of high-efficiency transformation and can be plated immediately onto agar/ampicillin plates following transformation.

The QIAGEN PCR Cloning Principle

QIAGEN PCR Cloning Kits take advantage of the single A overhang at each end of PCR products generated using *Taq* and other non-proofreading DNA polymerases. The pDrive Cloning Vector, supplied in a linear form with a U overhang at each end, hybridizes with high specificity to such PCR products. Optimal conditions for rapid and efficient hybridization and ligation are provided by the advanced buffer design of the Ligation Master Mix.

The QIAGEN PCR Cloning procedure is fast and easy — PCR products are simply mixed with pDrive Cloning Vector and Ligation Master Mix and then incubated at 4–16°C for 30 minutes (e.g., in a refrigerator). Special preparation of PCR products is not required. Transformation and plating using QIAGEN EZ Competent Cells takes only 10 minutes, making the total procedure, from PCR product to plating of transformed cells, just 40 minutes.

Product Description

pDrive Cloning Vector

The pDrive Cloning Vector is supplied in a linear form, ready-to-use for direct ligation of PCR products. This vector allows ampicillin and kanamycin selection, as well as blue/white colony screening. The vector contains several unique restriction endonuclease recognition sites around the cloning site, allowing easy restriction analysis of recombinant plasmids. The vector also contains a T7 and SP6 promoter on either side of the cloning site, allowing *in vitro* transcription of cloned PCR products as well as sequence analysis using standard sequencing primers. In addition, the pDrive Cloning Vector has a phage f1 origin to allow preparation of single-stranded DNA. A map of the pDrive Cloning Vector and the sequence of the region surrounding the cloning site are provided in Figure 1 (page 8). See “pDrive Cloning Vector restriction sites” (Appendix, page 24) for additional information.

Reagents

Ligation Master Mix, 2x: The Ligation Master Mix contains all reagents and cofactors required for ligation in a convenient pre-mixed format.

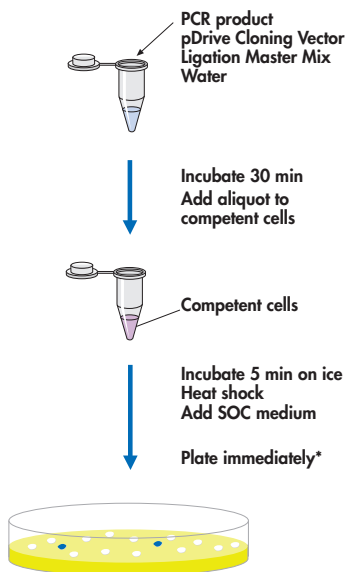
SOC medium: Tryptone, yeast extract, NaCl, KCl, glucose, MgCl_2 , MgSO_4 , sterile water

QIAGEN EZ Competent Cells

The genotype of QIAGEN EZ Competent Cells is: $[\text{F}'::\text{Tn10}(\text{Tc}^r)]$ proA^+B^- $\text{lacZ}\Delta\text{M15}$ recA1 endA1 $\text{hsdR17}(\text{r}_{\text{K12}}^- \text{m}_{\text{K12}}^+)$ lac glnV44 thi-1 gyrA96 relA1 (see Table 1, page 10, for further details).

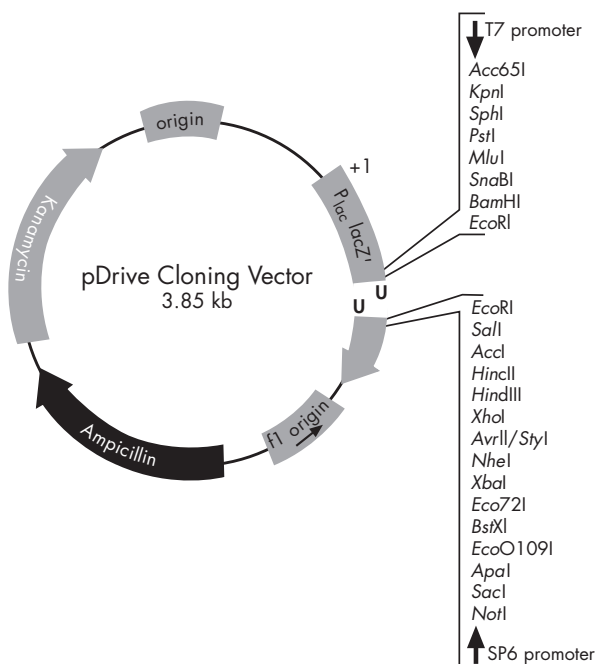
QIAGEN EZ Competent Cells are capable of high-efficiency transformation ($>1.0 \times 10^8$ colony forming units per microgram DNA), and are supplied in ready-to-use aliquots for single transformation reactions. The cells are compatible with ampicillin and kanamycin selection and blue/white screening following transformation with pDrive Cloning Vector. When using ampicillin selection, QIAGEN EZ Competent Cells do not require an incubation step following transformation, but instead can be directly plated.

QIAGEN PCR Cloning Kit Procedure



*Using QIAGEN EZ Competent Cells

A



B

Element	Position (bp)
Multiple cloning site	266–393
LacZ α -peptide	216–593
T7 RNA polymerase promoter	239–258
T7 transcription start	256
SP6 RNA polymerase promoter	398–417
SP6 transcription start	400
Ampicillin resistance gene	1175–2032
Kanamycin resistance gene	2181–2993
pUC origin	3668
Phage f1 origin	588–1043
Primer binding sites:*	
M13 forward (–20)	431–447
M13 forward (–40)	451–467
M13 reverse	209–224
T7 promoter primer	239–258
SP6 promoter primer	400–418

* Primer sequences are provided in the Appendix (page 27)

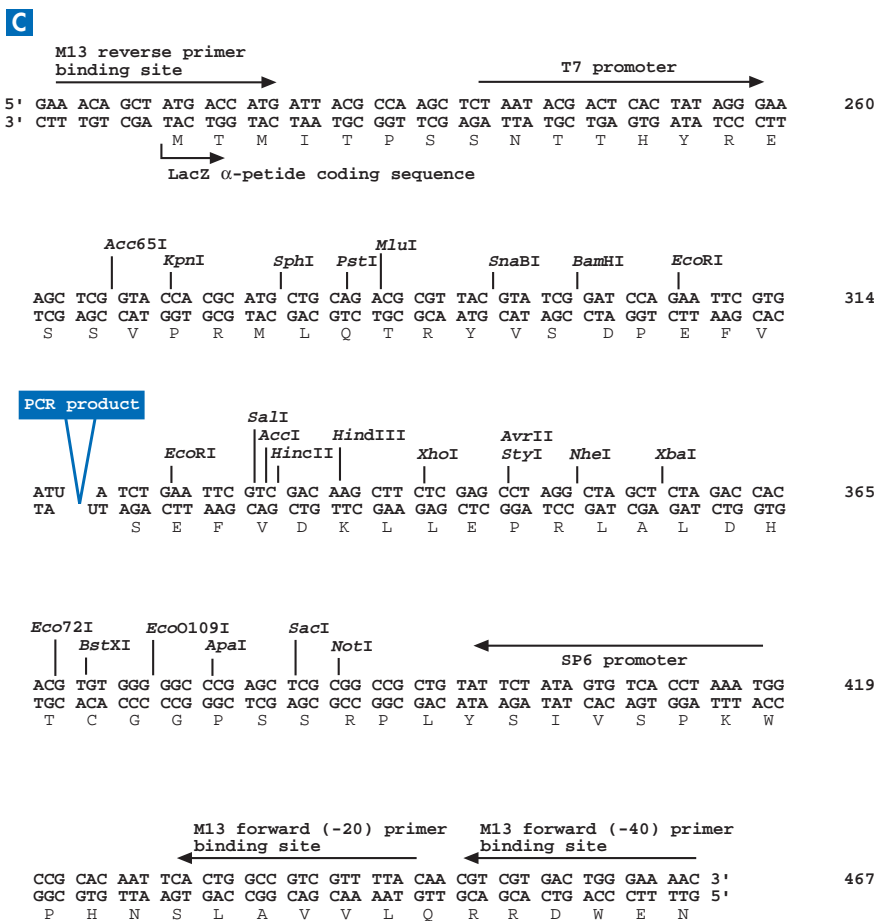


Figure 1. pDrive Cloning Vector Map. **A** Representation of the linearized pDrive Cloning Vector with U overhangs. The unique restriction endonuclease recognition sites on either side of the cloning site are listed. **B** Positions of various elements in the pDrive Cloning Vector. **C** DNA sequence of the region surrounding the cloning site. The amino acid sequence of the LacZ α-peptide is also given. The positions of the T7 and SP6 promoter sites and the M13 forward and reverse sequencing primer binding sites are provided, as are the cutting positions of the unique restriction endonucleases.

Table 1. Genotype of QIAGEN EZ Competent Cells

Genotype	Description/phenotype
<i>F'</i>	Presence of the low-copy-number F plasmid [<i>F'</i> :: <i>Tn10(Tc')</i> <i>proA</i> ⁺ <i>B</i> ⁺ <i>lacZ</i> Δ <i>M15</i>]
<i>Tn10(Tc')</i>	Transposon conferring tetracyclin resistance
<i>lac</i> ^P	Overproduction of the <i>lac</i> operon repressor, which controls expression from <i>P</i> _{lac}
<i>lacZ</i> Δ <i>M15</i>	Expression of N-terminally deleted β-galactosidase. This protein and LacZ α-peptide (encoded by the pDrive Cloning Vector) provide β-galactosidase activity. Cells transformed by pDrive Cloning Vector which does not contain a PCR product will express LacZ α-peptide, and will form blue colonies when grown in the presence of X-gal/IPTG. In contrast, cells transformed by pDrive Cloning Vector which does contain a PCR product will not express LacZ α-peptide, and will form white colonies.
<i>recA1</i>	Abolished homologous recombination
<i>end A1</i>	Abolished nonspecific endonuclease I activity, and hence improved DNA quality in plasmid preparations
<i>hsdR17</i>	This mutation prevents restriction, but not protective methylation, of unmethylated DNA or DNA containing foreign methylation patterns in <i>E. coli</i> cells.
<i>lac</i>	Inability to utilize lactose
<i>glnV44</i>	Suppressor of amber (UAG) mutation; required for growth of some phage vectors. Formerly called <i>supE</i> .
<i>thi-1</i>	Requires thiamin (vitamin B1) for growth in minimal medium
<i>gyrA96</i>	Mutation in DNA gyrase; resistance to naladixic acid
<i>relA1</i>	Relaxed mutation, permits RNA synthesis in the absence of protein synthesis

Quality Control

As part of the stringent QIAGEN quality assurance program, the performance of QIAGEN PCR Cloning Kits is monitored routinely and on a lot-to-lot basis. See the quality-control label inside the kit lid for lot-specific values.

pDrive Cloning Vector:

DNA contamination:	Plasmid DNA isolated from white colonies on a self-ligation plate is analyzed by agarose gel electrophoresis. "No" indicates that only plasmid corresponding to pDrive Cloning Vector was detected.
Exonuclease contamination:	Exonuclease contamination is tested by agarose gel electrophoresis. "No" indicates that exonuclease activity was not detected.
Cloning efficiency:	Cloning efficiency is tested using a PCR-generated control insert.

Reagents:

Ligation Master Mix, 2x:	Cloning efficiency is tested using a PCR-generated control insert. DNA contamination is tested by transforming QIAGEN EZ Competent Cells with Ligation Master Mix without plasmid DNA. "No" indicates that plasmid DNA contamination was not detected.
SOC medium:	Tested for sterility and performance in transformation.
Distilled water:	Tested for conductivity, pH, and sterility. Free of nucleases.

QIAGEN EZ Competent Cells:

DNA contamination:	Plasmid DNA contamination is tested by plating untransformed QIAGEN EZ Competent Cells. "No" indicates that DNA contamination was not detected.
Phage contamination:	Phage contamination is tested by plating QIAGEN EZ Competent Cells in top agar followed by visual inspection for plaques. "No" indicates that phage contamination was not detected.
Transformation efficiency:	Transformation efficiency is tested using a control plasmid in a transformation reaction. The transformation must yield $>1.0 \times 10^8$ colony forming units/ μ g control plasmid.

QIAGEN PCR Cloning Kit Ligation Protocol

Important notes before starting

- Use of PCR products generated with proofreading DNA polymerases (i.e., DNA polymerases with 3'-5' exonuclease activity) will dramatically lower ligation efficiency as these PCR products do not have an A overhang.
- We recommend using a molar ratio of 5–10 times more PCR product DNA than pDrive Cloning Vector DNA for ligation (Table 2). However, less PCR product may also be sufficient. PCR products can be concentrated using MinElute™ Kits.
- Purification of PCR products prior to ligation (e.g., using QIAquick® or MinElute PCR Purification or Gel Extraction Kits) is optional but recommended, as this will generally result in higher transformation efficiency. See "Purification of PCR products" (Appendix, page 23).
- The 5'-terminal base of the PCR primers can affect addition of an A overhang to PCR products by non-proofreading DNA polymerases. See "Effect of the 5'-terminal base of PCR products on cloning efficiency" (Appendix, page 21).
- Background colonies may appear following transformation if the PCR template was plasmid DNA containing a resistance gene for the antibiotic used for colony selection (i.e., an ampicillin- or kanamycin-resistance gene). In these cases the PCR product should be gel-purified prior to ligation to remove template plasmid DNA. Gel-purification can be avoided by using kanamycin for selection if the template plasmid contains the ampicillin-resistance gene, and vice versa.
- If **electrocompetent** cells will be used instead of QIAGEN EZ Competent Cells for transformation, we strongly recommend inactivating the ligase prior to electroporation by incubating the ligation-reaction mixture for 10 min at 70°C (see protocol step 4).

Table 2. Guide for the amount of PCR product to use in the ligation reaction

PCR product size	Amount of PCR product to use in the ligation reaction	
	5-times molar excess*	10-times molar excess*
100 bp	6.5 ng	13 ng
200 bp	13 ng	26 ng
500 bp	32.5 ng	65 ng
1000 bp	65 ng	130 ng
1500 bp	97.5 ng	195 ng
2000 bp	130 ng	260 ng
3000 bp	195 ng	390 ng

* Calculated for 50 ng pDrive Cloning Vector using the following equation:

$$\text{ng PCR product required} = \frac{50 \text{ ng} \times \text{PCR product size (bp)} \times \text{molar ratio}}{3851 \text{ bp}}$$

Procedure

1. **Thaw 2x Ligation Master Mix, pDrive Cloning Vector DNA, and distilled water (provided). Place on ice after thawing.**

It is important to mix the solutions completely before use to avoid localized concentrations of salts. Keep 2x Ligation Master Mix on ice and immediately store at -20°C or -70°C after use.

2. **Prepare a ligation-reaction mixture according to the following scheme:**

Component	Volume/reaction
pDrive Cloning Vector (50 ng/ μl)	1 μl
PCR product	1–4 μl *
Distilled water	variable
Ligation Master Mix, 2x [†]	5 μl
Total volume	10 μl

* Purified PCR product. If using non-purified PCR product, do not add more than 2 μl PCR product.

[†] We recommend adding the Ligation Master Mix last.

3. **Briefly mix the ligation-reaction mixture then incubate for 30 min at $4\text{--}16^{\circ}\text{C}$ (e.g., in a refrigerator, water bath, or thermal cycling block).**

Mix gently, for example by pipetting the ligation-reaction mixture up and down a few times.

Note: Increasing the ligation time to 2 h can result in a 2–3 fold increase of recombinants. This might be especially useful for PCR fragments longer than 2 kb. If the total number of recombinants is not essential, however, the ligation time can be as short as 15 min.

4. **Proceed with the transformation protocol (page 14) or store ligation-reaction mixture at -20°C until use.**

IMPORTANT: The transformation protocol on page 14 is for use with QIAGEN EZ Competent Cells. If **electrocompetent** cells will be used, we **strongly recommend** inactivating the ligase in the ligation-reaction mixture prior to electroporation. Incubate the ligation-reaction mixture for 10 min at 70°C , then proceed with electroporation. Alternatively, the MinElute Reaction Cleanup Kit can be used to remove ligase from the ligation-reaction mixture. The ligase does not need to be inactivated when using QIAGEN EZ Competent Cells.

QIAGEN PCR Cloning^{plus} Kit Transformation Protocol

Important notes before starting

- This protocol is for use with QIAGEN EZ Competent Cells. It is not for use with electrocompetent cells. If electrocompetent cells will be used, we **strongly recommend** inactivating the ligase in the ligation-reaction mixture prior to electroporation. See step 4 of the ligation protocol (page 13) for details.
- Competent cells are extremely sensitive to temperature and mechanical stress. **Do not allow QIAGEN EZ Competent Cells to thaw at any point prior to transformation.** Keep thawed cells on ice. Avoid excessive and/or rough handling, especially pipetting. Mix cells by **gentle** flicking.
- Thaw SOC medium and warm to room temperature. Store at -20°C or -70°C after use.
- Prepare fresh LB agar plates containing either ampicillin (100 $\mu\text{g}/\text{ml}$ LB agar) or kanamycin (30 $\mu\text{g}/\text{ml}$ LB agar) as a selection marker. Include IPTG (50 μM) and X-gal (80 $\mu\text{g}/\text{ml}$) for blue/white screening of recombinant colonies. See Appendix (page 27) for recipes.

Procedure

1. **Thaw the appropriate number of tubes of QIAGEN EZ Competent Cells on ice. Thaw SOC medium and warm to room temperature.**

IMPORTANT: Competent cells should only be thawed on ice. Do not allow unused QIAGEN EZ Competent Cells to thaw. Test whether cells are thawed by gently flicking the tube. Proceed immediately to the transformation step once the cells have thawed.

2. **Add 1–2 μl ligation-reaction mixture per tube of QIAGEN EZ Competent Cells, mix gently, and incubate on ice for 5 min.**

Mix gently, for example by flicking the transformation mixture a few times.

3. **Heat the tube(s) in a 42°C water bath or heating block for 30 s without shaking.**
4. **Incubate the tube(s) on ice for 2 min.**
5. **Add 250 μl room temperature SOC medium per tube and directly plate 100 μl each transformation mixture onto LB agar plates containing ampicillin.**

Note: For **kanamycin** selection, incubate the cells at 37°C for 30 min with shaking prior to plating to allow recombinant outgrowth.

The transformation mixture can be plated using a sterile bent glass rod or a specialized spreader. It is generally recommended to plate different amounts of each transformation mixture onto separate plates (e.g. 100 μl and 20 μl) to ensure good separation of colonies for subsequent single-colony isolation. For more efficient plating of small volumes of transformation mixture (<50 μl) we recommend pipetting 100 μl LB medium onto the plate, and then pipetting the transformation mixture into the liquid LB.

6. Incubate the plate at room temperature until the transformation mixture has absorbed into the agar. Invert the plate and incubate at 37°C overnight (e.g., 15–18 h).

Note: For blue/white screening, we recommend a second incubation at 4°C (e.g., in a refrigerator) for a few hours. This “cold” incubation step enhances blue color development and thereby facilitates differentiation between blue colonies and white colonies.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. Comments and suggestions are listed in the order in which they should be considered. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information or protocol in this handbook, or molecular biology applications in general (see inside front cover for contact information).

Observation	Possible cause	Comments and suggestions
No colonies on agar plate	a) Missing reagent	Check that all reagents were included in the ligation reaction and that a portion of the ligation mixture was added to the competent cells prior to transformation.
	b) Low transformation efficiency of competent cells	Use highly competent cells ($>1.0 \times 10^8$ cfu/ μ g circular plasmid DNA). If not using QIAGEN EZ Competent cells, test transformation efficiency by transforming the cells using a defined amount of circular plasmid DNA.
	c) Incorrect transformation procedure	Make sure that the appropriate transformation procedure was used, i.e., heat shock at 42°C for chemically competent cells (such as QIAGEN EZ Competent Cells) or electroporation for electrocompetent cells.
	d) Ligase was not inactivated prior to electroporation	If electrocompetent cells are used for transformation, we strongly recommend inactivating the ligase in the ligation-reaction mixture prior to electroporation. Following ligation, incubate the ligation-reaction mixture for 10 min at 70°C, then proceed with electroporation. Alternatively, MinElute Reaction Cleanup Kits can be used to remove ligase from the ligation-reaction mixture.
	e) Incorrect antibiotic	Check that the LB agar plate used contained the appropriate antibiotic.
	f) Incorrect incubation temperature(s)	Ensure that all incubation steps were performed at the correct temperature.

Observation	Possible cause	Comments and suggestions
	g) Incorrect DNA polymerase used for PCR	QIAGEN PCR Cloning Kits are designed for cloning of PCR products generated by non-proofreading DNA polymerases (e.g., <i>Taq</i> DNA polymerase). Proofreading DNA polymerases generate predominantly blunt-ended PCR products, which will not efficiently ligate to the pDrive Cloning Vector. An A overhang can be added to such PCR products by treatment with an appropriate polymerase.
Low number of white colonies	a) Incorrect DNA polymerase used for PCR	QIAGEN PCR Cloning Kits are designed for cloning of PCR products generated by non-proofreading DNA polymerases (e.g., <i>Taq</i> DNA polymerase). Proofreading DNA polymerases generate predominantly blunt-ended PCR products, which will not efficiently ligate to the pDrive Cloning Vector. An A overhang can be added to such PCR products by treatment with an appropriate polymerase.
	b) Inappropriate insert:vector ratio	Quantitate the PCR product by agarose gel analysis (see "Quantitation of PCR products", Appendix, page 23). We recommend a molar ratio of insert to vector of 5:1 to 10:1. In rare cases further optimization of the insert:vector ratio may be necessary.
	c) Ligation time too short	Increasing the ligation time can yield higher numbers of recombinant colonies.
	d) Inefficient addition of a single A overhang by the DNA polymerase	For efficient addition of an A overhang during the PCR procedure, we recommend a final extension step for at least 10 min. The efficiency of addition of a single A to PCR products by DNA polymerases is also sequence dependent. See "Effect of the 5'-terminal base of PCR primers on cloning efficiency", Appendix, page 21.
	e) Presence of inhibitor in PCR mixture	Purify the PCR product prior to ligation, e.g., using the QIAquick or MinElute systems. See "Purification of PCR products", Appendix, page 23.

Observation	Possible cause	Comments and suggestions
Only white colonies on the plate	f) Overexposure of PCR product to UV light	For gel-purification, use long-wave UV light to visualize PCR products and ensure that the DNA is exposed for as short a time as possible. Overexposure of DNA to UV induces pyrimidine dimers which reduce the efficiency of the procedure.
	g) Incorrect ligation temperature	Ensure that the temperature used for ligation does not exceed 16°C. Higher temperatures, e.g., greater than 25°C, give rise to increased background and fewer recombinants.
	h) Nuclease contamination	Nucleases may degrade the U overhang. Use only the provided distilled water and Ligation Master Mix, which are tested for absence of nucleases, in the ligation reaction.
	a) Inappropriate bacterial strain used for blue/white screening	Ensure that the bacterial strain used for transformation has a <i>lacZ</i> ΔM15 genotype. This mutation, which is present in QIAGEN EZ Competent Cells, is usually located on the F' plasmid and is a prerequisite for blue/white screening.
	b) Insufficient/no X-gal and/or IPTG	Check that the agar plates contain sufficient X-gal/IPTG. Use freshly prepared plates.
	c) Cells transformed with residual plasmid DNA from PCR reaction	Background colonies may appear following transformation if the PCR template was plasmid DNA containing a resistance gene for the antibiotic used for colony selection (i.e., an ampicillin- or kanamycin-resistance gene). In these cases the PCR product should be gel-purified prior to ligation to remove template plasmid DNA, e.g., using the QIAquick or MinElute systems. Gel purification can be avoided by using kanamycin for selection if the template plasmid contains the ampicillin-resistance gene, and vice versa. Alternatively, screen ampicillin-resistant white colonies for kanamycin resistance, and vice versa.

Observation	Possible cause	Comments and suggestions
Only blue colonies on the plate	d) Degraded antibiotic	The antibiotic present in the agar plate may have lost its full activity, allowing untransformed colonies to grow. Use freshly prepared plates. Add antibiotic to cooled agar (<55°C) before pouring plates.
	a) Inappropriate bacterial strain used for blue/white screening	Ensure that the bacterial strain used for transformation carries a <i>lacZ</i> mutation (e.g., QIAGEN EZ Competent Cells). <i>LacZ</i> ⁺ strains are not appropriate for blue/white screening.
	b) Functional LacZ α -peptide fusion protein	In rare cases the cloned PCR product will be expressed as a fusion protein with functional LacZ α -peptide. The length of such cloned fragments is usually a multiple of 3 bases (including the 3'-A overhang).
	c) Nuclease contamination	Nucleases may degrade the U overhang. Use only the provided distilled water and Ligation Master Mix, which are tested for absence of nucleases, in the ligation reaction.
	d) PCR product not added to the ligation-reaction mixture	Ensure that the PCR product was added to the ligation-reaction mixture.
Clones contain the PCR product in only one orientation		Expression of the PCR product in the appropriate reading frame (e.g., as a fusion protein with LacZ α -peptide) might be toxic for the cells. Omitting IPTG from the growth medium will decrease the expression level from P _{lac} , and therefore potentially allow the cells to survive. However, blue/white selection is usually not possible in the absence of IPTG.
White colonies do not contain the PCR product of interest	a) Cells transformed with residual plasmid DNA from PCR reaction	Background colonies may appear following transformation if the PCR template was plasmid DNA containing a resistance gene for the antibiotic used for colony selection (i.e., an ampicillin- or kanamycin-resistance gene). In these cases the PCR product should be gel-purified prior to ligation to remove template plasmid DNA, e.g., using the QIAquick or MinElute systems. Gel purification can be avoided by using kanamycin

Observation	Possible cause	Comments and suggestions
		for selection if the template plasmid contains the ampicillin-resistance gene, and vice versa. Alternatively, screen ampicillin-resistant white colonies for kanamycin resistance, and vice versa.
	b) Non-specific PCR products or primer-dimers cloned into pDrive Cloning Vector	Increase the stringency of the PCR conditions, for example by increasing the annealing temperature or using a hot-start procedure (e.g., HotStarTaq™ DNA Polymerase). Alternatively, gel-purify the PCR product prior to ligation.
	c) Nuclease contamination	Nucleases may degrade the U overhang. Use only the provided distilled water and Ligation Master Mix, which are tested for absence of nucleases, in the ligation reaction.

Appendix

Generation of PCR products

PCR products that will be cloned using QIAGEN PCR Cloning Kits should be generated using a thermostable DNA polymerase that does not possess proofreading activity, such as *Taq* DNA polymerase. Such polymerases attach a single A overhang to their reaction products, which can hybridize to the U overhang of the pDrive Cloning Vector. For efficient addition of an A overhang during the PCR procedure, we recommend a final extension step for 10 min at 72°C (as described in the PCR protocols for QIAGEN *Taq* DNA Polymerase and HotStarTaq DNA Polymerase).

High-specificity PCR is also a prerequisite for fast and easy cloning of PCR products. QIAGEN *Taq* DNA Polymerase and HotStarTaq DNA Polymerase are provided with innovative QIAGEN PCR Buffer, which supports specific primer annealing in each PCR cycle without the need for tedious optimization procedures. The use of a hot-start procedure can further increase PCR specificity. HotStarTaq DNA Polymerase, a modified form of QIAGEN *Taq* DNA Polymerase, provides high specificity in hot-start PCR. HotStarTaq DNA Polymerase is supplied in an inactive state that has no polymerase activity at ambient temperatures. This prevents extension of nonspecifically annealed primers and primer-dimers formed at low temperatures during PCR setup and the initial PCR cycle. HotStarTaq DNA Polymerase is activated by a 15-minute incubation at 95°C that can be easily incorporated into any existing thermal-cycler program. For more information about QIAGEN *Taq* DNA Polymerase or HotStarTaq DNA Polymerase, please call your local QIAGEN Technical Service Department or distributor (see inside front cover for contact information).

Effect of the 5'-terminal base of PCR primers on cloning efficiency

Thermostable DNA polymerases possessing no proofreading activity, such as *Taq* DNA polymerase, commonly add a single deoxynucleotide to the 3'-terminus of PCR products. Although deoxyadenosine (A) is generally added, this activity may also be dependent on the 5'-terminal base(s) of the primers used for PCR. The highest cloning efficiency will be obtained using PCR primers with a 5'-terminal deoxyadenosine.

General guidelines for PCR primer design and usage

Prerequisites for successful PCR include the design of optimal primer pairs, the use of appropriate primer concentrations, and the correct storage of primer solutions. Some general guidelines are provided in Table 3 (page 22; see also QIAGEN News 1997 No. 5, 1).

Table 3. General guidelines for standard PCR primers

Length:	18–30 nucleotides																
G/C content:	40–60%																
T_m:	<p>Simplified formula for estimating melting temperature (T_m): $T_m = 2^{\circ}\text{C} \times (\text{A}+\text{T}) + 4^{\circ}\text{C} \times (\text{G}+\text{C})$</p> <p>Whenever possible, design primer pairs with similar T_m values. Optimal annealing temperatures may be above or below the estimated T_m. As a starting point, use an annealing temperature 5°C below T_m.</p>																
Sequence:	<ul style="list-style-type: none"> • Avoid complementarity of two or three bases at the 3' ends of primer pairs to reduce primer–dimer formation. • Avoid mismatches between the 3' end of the primer and the target-template sequence. • Avoid runs of 3 or more G or C at the 3' end. • Avoid a 3'-end T. Primers with a T at the 3' end have a greater tolerance of mismatch. • Avoid complementary sequences within a primer sequence and between the primer pair. • Commercially available computer software (e.g., Primer Designer 1.0, Scientific Software, 1990; Oligo, Rychlik and Rhoads, 1989) can be used for primer design. 																
Concentration:	<ul style="list-style-type: none"> • Spectrophotometric conversion for primers: $1 A_{260} \text{ unit} = 20\text{--}30 \mu\text{g/ml}$ • Molar conversions: <table> <tr> <th>Primer length</th><th>pmol/μg</th><th>20 pmol</th></tr> <tr> <td>18mer</td><td>168</td><td>119 ng</td></tr> <tr> <td>20mer</td><td>152</td><td>132 ng</td></tr> <tr> <td>25mer</td><td>121</td><td>165 ng</td></tr> <tr> <td>30mer</td><td>101</td><td>198 ng</td></tr> </table>		Primer length	pmol/ μg	20 pmol	18mer	168	119 ng	20mer	152	132 ng	25mer	121	165 ng	30mer	101	198 ng
Primer length	pmol/ μg	20 pmol															
18mer	168	119 ng															
20mer	152	132 ng															
25mer	121	165 ng															
30mer	101	198 ng															
Storage:	<ul style="list-style-type: none"> • Use 0.1–0.5 μM of each primer in PCR. For most applications, a primer concentration of 0.2 μM will be sufficient. <p>Lyophilized primers should be dissolved in a small volume of distilled water or TE to make a concentrated stock solution. Prepare small aliquots of working solutions containing 10 pmol/μl to avoid repeated thawing and freezing. Store all primer solutions at -20°C. Primer quality can be checked on a denaturing polyacrylamide gel; a single band should be seen.</p>																

Purification of PCR products

After amplification, the PCR sample contains a complex mixture of specific PCR product and residual reaction components such as primers, unincorporated nucleotides, enzyme(s), salts, mineral oil, and possibly non-specific amplification products. Removal of these contaminants may increase cloning efficiency. The QIAquick system offers a quick and easy method for purifying PCR products. The MinElute system should be used if the PCR product needs to be concentrated, e.g., due to inefficient PCR. See page 30 for ordering information. For more information about QIAquick or MinElute Kits, please call your local QIAGEN Technical Service Department or distributor (see inside front cover for contact information).

Quantitation of PCR products

DNA concentration is generally determined either by spectrophotometric measurement of the adsorption at 260 nm or by agarose gel analysis. Absorbance readings (A_{260}) should fall between 0.1 and 1.0 to be accurate. Sample dilution should be adjusted accordingly. An absorbance of 1.0 at 260 nm corresponds to 50 µg DNA/ml. Since spectrophotometric measurement cannot differentiate between PCR products, primers, and oligonucleotides, the PCR product should be purified prior to measurement. The QIAquick and MinElute systems offer quick and easy methods for purifying PCR products (see page 30 for ordering information).

PCR products can be quickly and easily quantitated by agarose gel analysis. The sample is run in an agarose gel side-by-side with known amounts of DNA of the same size (e.g., an appropriate DNA ladder). The DNA can be visualized under UV light following staining with ethidium bromide*, and the DNA concentration of the sample estimated by visual comparison of the intensity of the sample band with that of the standards. More precise quantitations can be obtained by densitometric measurement of band intensity and comparison with a standard curve.

Preparation of recombinant plasmid DNA

Many downstream applications require high-purity or ultrapure DNA. QIAGEN offers a complete range of plasmid preparation systems ensuring the highest quality plasmid DNA, for example, the QIAprep® miniprep system for fast and simple preparation of up to 20 µg high-purity DNA, and the HiSpeed™ Plasmid Kit for fast purification of ultrapure DNA. See page 30 for ordering information. For more information about plasmid purification products, please call your local QIAGEN Technical Service Department or distributor (see inside front cover for contact information).

* Toxic and/or mutagenic. Take appropriate safety measures.

pDrive Cloning Vector restriction sites

The pDrive Cloning Vector is supplied in a linear form with a U overhang at each end. Insertion of a PCR product destroys the *EcoRV* site of the vector, and therefore use of *EcoRV* for restriction mapping of recombinant plasmid is not recommended. The first base of the *lacZ* 5' upstream region is designated as nucleotide number one. Table 4 lists restriction enzymes that do not cut the pDrive Cloning Vector, and Table 5 lists restriction enzymes that cut the vector 1–5 times. The complete pDrive Cloning Vector sequence is available on the QIAGEN web site (www.qiagen.com/literature/vectors.asp).

Table 4. Restriction enzymes that do not cut the pDrive Cloning Vector

<i>Aat</i> II	<i>Bgl</i> II	<i>Bss</i> HII	<i>Dsa</i> I	<i>Nco</i> I	<i>Sfi</i> I
<i>Acc</i> II	<i>Bpl</i> I	<i>Bst</i> 1107I	<i>Eco</i> 47III	<i>Nde</i> I	<i>Sgr</i> AI
<i>Age</i> I	<i>Bse</i> RI	<i>Bst</i> 98I	<i>Ehe</i> I	<i>Pac</i> I	<i>Spe</i> I
<i>Asc</i> I	<i>Bsg</i> I	<i>Bst</i> API	<i>Fse</i> I	<i>Pin</i> AI	<i>Srf</i> I
<i>Bal</i> I	<i>Bsi</i> WI	<i>Bst</i> EII	<i>Hpa</i> I	<i>Pme</i> I	<i>Sse</i> 8387I
<i>Bbe</i> I	<i>Bsm</i> FI	<i>Bst</i> HPI	<i>Kas</i> I	<i>Ppu</i> MI	<i>Stu</i> I
<i>Bbs</i> I	<i>Bsp</i> 19I	<i>Bsu</i> 36I	<i>Mfe</i> I	<i>Psh</i> AI	<i>Swa</i> I
<i>Bbv</i> CI	<i>Bsp</i> MI	<i>Btr</i> I	<i>Msc</i> I	<i>Sac</i> II	<i>Tth</i> 1111I
<i>Bcl</i> I	<i>Bsr</i> BRI	<i>Csp</i> I	<i>Mun</i> I	<i>San</i> DI	<i>Xcm</i> I
<i>Bcu</i> I	<i>Bsr</i> GI	<i>Csp</i> 45I	<i>Nar</i> I	<i>Sex</i> AI	

Table 5. Restriction enzymes that cut the pDrive Cloning Vector 1–5 times

Enzymes that cut once					
<i>AccI</i>	328*	<i>Eco31I</i>	1896	<i>PspAI</i>	2484
<i>Acc65I</i>	266	<i>Eco52I</i>	387	<i>PspOMI</i>	374
<i>Adel</i>	812	<i>Eco72I</i>	367	<i>PstI</i>	283
<i>AlwNI</i>	3316	<i>EcoCR1</i>	382	<i>SacI</i>	384
<i>Apal</i>	378	<i>EcoNI</i>	2524	<i>SalI</i>	327
<i>AvrII</i>	345	<i>EcoO109I</i>	374	<i>SapI</i>	3842
<i>BamHI</i>	299	<i>EcoRV[†]</i>	316	<i>Scal</i>	1481
<i>BcgI</i>	1424	<i>Esp3I</i>	2628	<i>SgfI</i>	2612
<i>Bpml</i>	1893	<i>FblI</i>	328	<i>SmaI</i>	2486
<i>Bpu10I</i>	2629	<i>HincII</i>	329	<i>SnaBI</i>	293
<i>Bsal</i>	1896	<i>HindIII</i>	333	<i>SphI</i>	278
<i>BsmBI</i>	2628	<i>Hsp92I</i>	1422	<i>SstI</i>	384
<i>BspDI</i>	2303	<i>KpnI</i>	270	<i>StyI</i>	345
<i>BspLU11I</i>	3725	<i>MluI</i>	285	<i>Van91I</i>	2875
<i>BstXI</i>	369	<i>NaeI</i>	915	<i>XbaI</i>	356
<i>CciNI</i>	387	<i>NgoMIV</i>	913	<i>XhoI</i>	339
<i>Cfr9I</i>	2484	<i>NheI</i>	350	<i>XmaI</i>	2484
<i>Clal</i>	2303	<i>NotI</i>	387	<i>XmaIII</i>	387
<i>DraIII</i>	812	<i>NruI</i>	2269		
<i>EclHKI</i>	1962	<i>PsiI</i>	684		
Enzymes that cut twice					
<i>Alw44I</i>	1290, 3411	<i>BsrDI</i>	1728, 1902	<i>NspI</i>	278, 3729
<i>ApalI</i>	1290, 3411	<i>BssSI</i>	1293, 3552	<i>Ppu10I</i>	2458, 2724
<i>Avall</i>	1598, 1820	<i>DrdI</i>	767, 3623	<i>Psp1406I</i>	1360, 1733
<i>BciVI</i>	1125, 3527	<i>Eco47I</i>	1598, 1820	<i>PvuII</i>	54, 517
<i>BglI</i>	577, 1844	<i>Eco57I</i>	1296, 3183	<i>SinI</i>	1598, 1820
<i>Bmrl</i>	456, 1911	<i>EcoRI</i>	306, 321	<i>TatI</i>	1478, 1484
<i>BsaMI</i>	2496, 2573	<i>FspI</i>	567, 1739	<i>XmnI</i>	1362, 3001
<i>BseMI</i>	1728, 1902	<i>NsiI</i>	2462, 2728		

(cont. page 26)

Enzymes that cut three times

<i>Alw26I</i>	1120, 1896, 2628	<i>BsrFI</i>	913, 1877, 2566	<i>PvuI</i>	548, 1593, 2612
<i>AvaI</i>	339, 377, 2484	<i>Cfr10I</i>	913, 1877, 2566	<i>RsaI</i>	268, 1481, 2447
<i>BsaAI</i>	293, 367, 812	<i>Csp6I</i>	267, 1480, 2446	<i>SspI</i>	604, 1157, 2537
<i>BsmAI</i>	1120, 1896, 2628	<i>DraI</i>	1384, 2076, 2095		
<i>BspHI</i>	1122, 2130, 3005	<i>Eco88I</i>	339, 377, 2484		

Enzymes that cut four times

<i>AflIII</i>	285, 364, 366, 3725	<i>BsiHKAII</i>	384, 1294, 1379, 3415	<i>Eco64I</i>	130, 266, 849, 2009
<i>BanI</i>	130, 266, 849, 2009	<i>BsrBI</i>	186, 956, 1120, 3796	<i>HaeII</i>	4, 963, 971, 3485
<i>BanII</i>	378, 384, 887, 2267	<i>EarI</i>	526, 1163, 2425, 3842	<i>MbiI</i>	186, 956, 1120, 3796
<i>BsaWI</i>	1666, 2747, 3372, 3519	<i>Eco24I</i>	378, 384, 887, 2267		

Enzymes that cut five times

<i>Asel</i>	46, 105, 1787, 2811, 3000	<i>FauI</i>	34, 76, 546, 949, 1018	<i>NciI</i>	1426, 1777, 2485, 2486, 3348
<i>BcnI</i>	1426, 1777, 2485, 2486, 3348	<i>HgaI</i>	293, 1029, 1430, 3035, 3613	<i>SmlI</i>	339, 1337, 3080, 3357, 3619
<i>EaeI</i>	35, 387, 417, 433, 1569	<i>MslI</i>	367, 413, 1191, 1550, 1709	<i>VspI</i>	46, 105, 1787, 2811, 3000

* Numbers refer to the position of the 3' base of the restricted DNA in each recognition sequence. The first base of the lacZ 5' upstream region of the pDrive Cloning Vector is designated as nucleotide number one.

† Insertion of a PCR product destroys the EcoRV site of the vector. Use of EcoRV for restriction mapping of recombinant plasmid is therefore not recommended.

Recipes

X-gal stock solution (40 mg/ml)

Dissolve 400 mg 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside in 10 ml dimethylformamide.* Protect the solution from light by wrapping in aluminium foil or by using a brown bottle. Store at -20°C .

IPTG stock solution (100 mM)

Dissolve 238.3 mg isopropyl β -D-thiogalactopyranoside in 10 ml deionized water. Filter-sterilize and store in aliquots at -20°C .

Ampicillin stock solution (100 mg/ml)

Dissolve 5 g ampicillin sodium salt in 50 ml deionized water. Filter-sterilize and store in aliquots at 4°C .

Kanamycin stock solution (30 mg/ml)

Dissolve 1.5 g kanamycin monosulfate salt in 50 ml deionized water. Filter-sterilize and store in aliquots at 4°C .

LB (Luria Bertani) agar plates (1000 ml = approx. 40 plates)

Dissolve 10 g tryptone, 5 g yeast extract, 10 g NaCl, and 15 g agar in 950 ml deionized water. Adjust the pH of the solution to 7.0 and add deionized water to 1000 ml. Autoclave the solution for 20 min.

To pour plates, allow autoclaved LB agar to cool to 55°C . Add 1 ml ampicillin stock solution and/or kanamycin stock solution, as appropriate. If blue/white screening will be performed, add 2 ml X-gal stock solution and 0.5 ml IPTG stock solution. Mix, pour agar into plates, allow to set, invert, and store at 4°C under sterile conditions.

We recommend using freshly poured plates (<1 week old) for plating cells. Long-term storage of plates can result in decreased antibiotic activity.

Primer sequences

M13 forward (–20): 5' GTAAAACGACGGCCAGT 3'

M13 forward (–40): 5' GTTTTCCCAGTCACGAC 3'

M13 reverse: 5' AACAGCTATGACCATG 3'

SP6 promoter: 5' CATTTAGGTGACACTATAG 3'

T7 promoter: 5' GTAATACGACTCACTATAG 3'

* Toxic and/or mutagenic. Take appropriate safety measures.

Ordering Information

Product	Contents	Cat. No.
QIAGEN PCR Cloning Kits — for direct cloning of PCR products		
QIAGEN PCR Cloning Kit (10)	For 10 reactions: 2x Ligation Master Mix (50 µl), pDrive Cloning Vector (0.5 µg), Distilled water (1.7 ml)	231122
QIAGEN PCR Cloning Kit (40)	For 40 reactions: 2x Ligation Master Mix (200 µl), pDrive Cloning Vector (2.0 µg), Distilled water (1.7 ml)	231124
QIAGEN PCR Cloning ^{plus} Kit (10)	For 10 reactions: 2x Ligation Master Mix (50 µl), pDrive Cloning Vector (0.5 µg), Distilled water (1.7 ml), QIAGEN EZ Competent Cells (10 tubes, 50 µl each), SOC medium (2 x 1.9 ml)	231222
QIAGEN PCR Cloning ^{plus} Kit (40)	For 40 reactions: 2x Ligation Master Mix (200 µl), pDrive Cloning Vector (2.0 µg), Distilled water (1.7 ml), QIAGEN EZ Competent Cells (40 tubes, 50 µl each), SOC medium (6 x 1.9 ml)	231224

Related products for PCR

HotStarTaq DNA Polymerase — for highly specific hot-start PCR

HotStarTaq DNA Polymerase (250)*	250 units HotStarTaq DNA Polymerase, 10x PCR Buffer, [†] 5x Q-Solution, 25 mM MgCl ₂	203203
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QIAGEN Taq DNA Polymerase — for standard and specialized PCR applications

Taq DNA Polymerase (250)*	250 units Taq DNA Polymerase, 10x PCR Buffer, [†] 5x Q-Solution, 25 mM MgCl ₂	201203
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Ordering Information

Product	Contents	Cat. No.
QIAGEN OneStep RT-PCR Kit — for fast and efficient one-step RT-PCR		
QIAGEN OneStep RT-PCR Kit (25)*	For 25 one-step RT-PCRs: QIAGEN OneStep RT-PCR Enzyme Mix, 5x QIAGEN OneStep RT-PCR Buffer, [‡] dNTP Mix, [§] 5x Q-Solution, RNase-free water	210210
Omniscript RT Kit — for standard reverse transcription		
Omniscript RT Kit (10)*	For 10 reverse-transcription reactions: 40 units Omniscript Reverse Transcriptase, 10x Buffer RT, dNTP Mix, [¶] RNase-free water	205110
Sensiscript RT Kit — for reverse transcription using <50 ng RNA		
Sensiscript RT Kit (50)*	For 50 reverse-transcription reactions: Sensiscript Reverse Transcriptase, 10x Buffer RT, dNTP Mix, [¶] RNase-free water	205211

* Larger kit sizes available

[†] Contains 15 mM MgCl₂

[‡] Contains 12.5 mM MgCl₂

[§] Contains 10 mM each dNTP

[¶] Contains 5 mM each dNTP

Ordering Information

Product	Contents	Cat. No.
Related products for PCR product cleanup		
QIAquick PCR Purification Kit — for direct purification of PCR fragments		
QIAquick PCR Purification Kit (50)*	50 QIAquick Spin Columns, Buffers, Collection Tubes (2 ml)	28104
QIAquick Gel Extraction Kit — for gel extraction of DNA fragments		
QIAquick Gel Extraction Kit (50)*	50 QIAquick Spin Columns, Buffers, Collection Tubes (2 ml)	28704
MinElute PCR Purification Kit — for purification of PCR products in low elution volumes		
MinElute PCR Purification Kit (50)*	50 MinElute Spin Columns, Buffers, Collection Tubes (2 ml)	28004
MinElute Gel Extraction Kit — for gel extraction of DNA fragments in low elution volumes		
MinElute Gel Extraction Kit (50)*	50 MinElute Spin Columns, Buffers, Collection Tubes (2 ml)	28604
MinElute Reaction Cleanup Kit — for cleanup of DNA from enzymatic reactions		
MinElute Reaction Cleanup Kit (50)*	50 MinElute Spin Columns, Buffers, Collection Tubes (2 ml)	28204
Related products for plasmid DNA preparation		
QIAprep Miniprep Kits — for high-purity plasmid minipreps		
QIAprep Spin Miniprep Kit (50)*	50 QIAprep Spin Columns, Reagents, Buffers, Collection Tubes (2 ml)	27104
HiSpeed Plasmid Kit — for ultrafast purification of plasmid or cosmid DNA		
HiSpeed Plasmid Midi Kit (25)*	25 HiSpeed Midi Tips, 25 QIAfilter Midi Cartridges, 25 QIAprecipitator Modules plus Syringes, Reagents, Buffers	12643

* Larger kit sizes and/or different kit formats available

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